REVIEW

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Progress towards gene therapy for HIV infection

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The retroviral life cycle and genetic plasticity of human immunodeficiency virus 1 (HIV-1) present unprecedented therapeutic challenges. Twelve years into the HIV epidemic, satisfactory treatment remains elusive. Our current understanding of AIDS pathogenesis calls for early intervention with antiviral agents. Although still in its infancy, human gene therapy holds considerable potential for the long-term treatment of genetic disorders, cancer and chronic infectious diseases. Gene therapy for HIV infection is receiving particularly intensive study: approaches that are in development include both immunotherapy (e.g. therapeutic vaccines and adoptive transfer of CD8+ T-cell clones) and direct antiviral therapy (intracellular immunization). The latter strategies include transdominant modifications of HIV proteins, RNA decoys, antisense RNA, ribozymes and modifications of cellular proteins (e.g. intracellular antibodies, soluble CD4). Several of these strategies are now entering clinical trials. While significant conceptual and technical hurdles remain to be overcome before the promise of gene therapy for HIV infection can be fully realized, progress in this field is likely to be rapid and to contribute to the broader applicability of human gene therapy to the treatment of other disorders.

Introduction

AIDS: an acquired genetic disease

The recognition that acquired immunodeficiency syndrome (AIDS) is caused by a retrovirus presented the research community with a formidable therapeutic challenge. Like other members of the family Retroviridae, human immunodeficiency virus-1 (HIV-1) subverts biology's central dogma by reverse-transcribing its RNA genome and randomly integrating the resulting double-stranded DNA into a chromosome of its host cell [1]. There the provirus becomes a heritable feature of the cell and retains the potential for viral expression and production of progeny virus for the duration of the cell's lifetime. An HIV-infected person becomes, in effect, genetically mosaic for HIV. In this sense, AIDS is a genetic disease, albeit an acquired one. The exceptional genetic plasticity of HIV provides additional layers of complexity. Rapidly changing viral populations (the evocative terms 'swarm' and 'quasi-species' are often applied) evolve within each patient while molecular epidemiological comparisons also reveal considerable interpatient variability as well as distinct genetic subtypes with particular geographic distributions [2]. Therapeutic strategies must ensure not only broad efficacy against diverse virus strains, but also prevention of the emergence of resistant viruses.

Scientific appreciation of the scale of the fundamental difficulties posed by these attributes of HIV-1 has been matched by commensurately bleak reports from the clinical front. Twelve years after the first description of AIDS, the global pandemic is expanding, and new epicenters, most notably South and Southeast Asia, have arisen [3]. Despite unprecedented progress in understanding the causative agent at the molecular level and in palliating the myriad complications of AIDS, HIV disease remains refractory to treatment. In 1993, death within several years is a virtual certainty for HIV-infected patients who have begun to suffer the opportunistic infections and malignancies of AIDS [4].

Previously studied therapeutic agents for HIV infection include reverse transcriptase inhibitors and soluble CD4 [5, 6]. Their limited efficacy may stem in part from the fact that they inhibit infection by pre-existing viral particles; infected cells can continue to generate viral progeny in patients who are taking these drugs. Such unabated virion production in turn sets the scene for the selection of escape mutants.

Gene transfer for HIV: prospects and problems

For all of these considerations, the still nascent field of gene therapy may hold considerable potential for the treatment of HIV infection. Indeed, this sense of promise has engendered a diversity of gene-transfer-based approaches to HIV therapy, encompassing direct antiviral strategies, as well as immunotherapy [7] and vaccines. However, many practical problems of gene delivery and expression await resolution. Several considerations limit our optimism for gene therapeutic strategy for AIDS as opposed to the initial gene therapy candidate diseases such as ADA (adenosine deaminase) deficiency. First is the lack of a secure understanding of AIDS pathogenesis. Despite triumphs of the molecular dissection of HIV, the enigma of the precise mechanism(s) of immune destruction still persists. Second, while the defect in ADA deficiency is recessive

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and can be corrected by the addition of a corrected allele [8], the HIV provirus is effectively an autosomal dominant gene. Targeting either the gene or its products will require a significant leap in sophistication in gene therapy. The ultimate goal, radical cure, for HIV therapy would require either the death (by active killing or by attrition) of the reservoir of infected cells or definitive disabling of all integrated proviruses. Some emerging strategies may, in principle, enable the killing of infected cells [7] but selective gene excision, using methods such as homologous recombination [9, 10], is still technically daunting.

Notwithstanding these reservations, the pace in research towards gene therapy for HIV infection has gained momentum, and a few approaches are on the brink of being evaluated in the clinic. This article reviews some of these developments and looks towards the future in this exciting arena.

Immunotherapy

Immunization by in vivo expression of viral proteins

Attempts to express HIV proteins *in vivo* for prophylactic vaccination or for modulation of the immune response of infected individuals may be the most immediately feasible uses of gene transfer for HIV infection. First, this approach can achieve *de novo* intracellular synthesis of viral proteins that mimics natural infection; viral protein-derived peptides can then be optimally presented to $CD8^+$ T lymphocytes in the context of class I major histocompatibility complex (MHC) molecules. Second, the strategy is not necessarily constrained by the twin challenges that usually confront the gene therapist: the attainment of stable, high level gene expression and the targeting of gene delivery to specific cell populations. Low level expression in most cell types can be sufficient to generate cell-mediated immune responses [11]; low dose antigen administration may in fact be preferable for the development of a T_H1 immune response [12]. Whether generation of cell-mediated immunity to HIV by such a route could be consistently protective, with or without concomitantly generated neutralizing antibody, is unknown.

For other viral infections, however, gene-transfer-based protective immunity has now been clearly documented in animal models. A recent notable example employed direct DNA injection, a method of gene delivery remarkable for both its simplicity and its serendipitous discovery [13]. Direct injection of saline containing only pure plasmid DNA encoding the influenza A nucleoprotein into muscle (without liposomes or other transfecting agents) resulted not only in persistent gene expression but also generation of antigen-specific cytotoxic T lymphocytes (CTLs) and protection from otherwise lethal challenge with influenza virus [14]. Moreover, use of the conserved internal nucleoprotein rather than the variable envelope protein circumvented the strain-specificity limitations of conventional influenza vaccines: the mice were resistant to challenge with a heterologous influenza strain arising 34 years after the vaccine strain.

This and other reports [13, 15] suggest that direct (or 'naked') DNA may offer the ability to mimic the immunogenically critical aspects of natural infection without the risks of a replicating vector or viral particles by achieving sustained intracellular, class I MHC-restricted presentation of peptides derived from endogenously synthesized viral proteins. In contrast, exogenous proteins (e.g. from conventional killed virus or recombinant protein vaccines) generally enter the endosomal processing pathway, are presented by class II MHC molecules and are unlikely to generate effective $CD8^+$ CTL responses [16]. The method also has other such potentially overwhelming advantages – simplicity, stability, economy – that its application for prophylaxis and therapy of HIV infection is likely to progress rapidly.

Weiner and colleagues recently vaccinated mice with HIV-1 sequences by direct DNA injection [17]. The mice were given four biweekly intramuscular injections of 100 μg of an HIV-1 (HXB2) gp160-expressing plasmid. They developed homologous HIV-1 strain-specific neutralizing antibodies and lymphocyte proliferation responses. Numerous questions that await clarification include the nature of the antigen presenting cells, the optimal choice of immunogens, the extent of interstrain cross-reactivity and the degree of protection conferred to non-human primates [18] and eventually humans against natural challenge.

Conventional retroviral vector delivery of HIV-1 genes for vaccine and immunomodulating purposes is also investigated. In pre-clinical studies of a gene-transfer-based vaccine for HIV, Warner *et al.* [19, 20] used an MMLV-based retroviral vector to deliver a gene encoding HIV-IIIB gp160 to murine fibroblast cell lines. Mice immunized with syngeneic transduced cells developed MHC-restricted, envelope-specific CTLs; following adoptive cell transfer, these cells were capable of eliminating established tumor cells expressing the HIV-1 envelope protein. The mice also developed HIV-1 envelope-specific antibodies that inhibited syncytia formation in an HIV-infected human T-cell line. Recently, transduction of autologous cells yielded similar results in mice and in non-human primates without any evident toxicity [21].

Retroviral vector transfer of the gene encoding HIV-1 gp160 has now advanced to the clinical trial stage [22, 23] (see section on RAC-approved clinical protocols). However, the utility of 'therapeutic vaccines' in patients with HIV infection remains a murky issue. These individuals are immunosupressed to begin with; their ability to mount an effective response is naturally in doubt. Furthermore, intracellular expression of the same protein(s) by the native virus has failed by definition to yield an effective cellular immune response. On the other hand, immune recognition of proteins produced in HIV-1 infected cells may differ substantially from transduced cells. For example, viral interference with class I MHC antigen presentation, an immune evasion strategy amply documented for other viruses [24, 25] and to some extent for HIV [26–28], may not occur with isolated expression of single HIV-1 structural proteins or protein fragments.

Adoptive transfer of gene-altered CTLs

Studies of adoptive transfer of antigen-specific CD8+ T-cell clones for opportunistic cytomegalovirus (CMV) infection by Greenberg *et al.* have illuminated the potential of this approach for the treatment of HIV [29]. CMV is a major cause of morbidity and mortality in the immunosupressed [30]. During primary CMV infection, individuals develop CD8+ CTLs that recognize CMV antigen presented in association with class I MHC molecules [31]. Adoptive transfer of educated, expanded CMV-specific CD8+ T cells was first developed in murine models. Protection was afforded even when murine CMV-pneumonia was established before cell transfer [32]. Human investigations subsequently showed that clonally derived antigen-specific T cells can be generated and adoptively transferred to reconstitute specific human immune responses without toxicity [33–35]. In these studies, CMV-specific CD8+ T cell clones were produced from CMV-seropositive healthy donors by co-culture of peripheral blood lymphocytes (PBLs) from donors and patients with CMV-infected donor-derived fibroblasts. After cell transfer, the clones provided persistent reconstitution of CMV-specific CTL responses.

Similar studies utilizing HIV-1-specific CD8+ T-cell clones are being carried out. Although toxicity was not apparent in earlier studies, the investigators have proceeded to incorporate a 'suicide gene', a hybrid of the herpes simplex virus thymidine kinase gene (HSVtk) and the hygromicin resistance gene, into the vector used to mark antigen-specific CD8+ CTLs [36]. If undesirable effects of the infused cells become prohibitive, administration of ganciclovir to the host should result in HSVTK-catalyzed phosphorylation of the drug to its triphosphate form and selective killing of the gene-altered cells. Because a functioning gene is being transferred for an ancillary therapeutic purpose, this protocol properly falls under the rubric of gene therapy rather than gene marking. This was the first gene therapy proposal for the treatment of HIV infection to receive approval.

Antiviral therapy (intracellular immunization)

Transdominant HIV proteins

Background of concept of intracellular immunization. In 1988 McKnight and co-workers demonstrated antiviral effects of a truncated form of the herpes simplex virus transactivator protein VP16 [37]. The modified protein had a deletion in the transactivation domain but retained the native form's DNA-binding activity. Cells stably expressing this mutant did not support wild-type HSV replication, suggesting that it interfered with the occupation of a cis-acting element by wild-type viral protein [37]. The authors suggested that a similar strategy could be devised to inhibit HIV-1 Tat. In an accompanying commentary, David Baltimore coined the term 'intracellular immunization' to describe the design of this and other forms of gene-transfer-based cellular resistance to viral infection [38]. Table 1 summarizes the status of these approaches for HIV.

Table 1 Current anti-HIV gene therapy approaches[a]

Therapeutic gene products	*Efficacy in stable T-cell lines*	*Viral vector*	*Protection of primary PBL*	*Challenge with primary isolates*
Transdominant proteins				
Rev	+	MMLV	+	–[c]
Tat	–	–	–	–
Gag	+	MMLV	–	–
Env	+	–	–	–
Engineered cellular proteins				
sCD4	+	MMLV	–	–
sCD4-KDEL	+	MMLV	–	–
Intracellular Ab	–	–	–	–
RBP9-27	–	–	–	–
RNA inhibitors				
TAR and RRE decoys	+	MMLV	–	–
Antisense	+	MMLV, AAV	–	–
Hammerhead Rz	+	MMLV	–	–
Hairpin Rz	+	MMLV	+[b]	+

–, No published data available.
[a]see text for detail and specific references.
[b]Leavitt, M. *et al.*, unpublished results.
[c]Nabel, G., personal communication.

Regulatory proteins: Rev, Tat. For HIV-1, both structural (Gag, Env) and regulatory (Tat, Rev) transdominant mutant proteins have been studied, with the transdominant Rev protein being the most intensively investigated. Attention has been drawn to a well-conserved leucine-rich domain close to the C terminus which is absolutely essential for the function of Rev as a regulator of the stability and nuclear export of unspliced and singly-spliced viral mRNAs [39]. Mutations in this region yield defective proteins that act as transdominant inhibitors of wild-type Rev in transient transfection assays [40–42]. The RevM10 transdominant mutant, with amino acid substitutions of D for L and L for E at positions 78 and 79, respectively, retains the Rev response element (RRE) binding and protein multimerization properties of wild-type Rev [43,44], and can potentially multimerize with wild-type Rev and/or competitively inhibit RRE binding [44]. A MMLV-based retroviral vector was used to deliver RevM10 to human T cells [45]. Cloned human CEM cell lines stably expressing RevM10 were resistant to viral infection. Transduction of primary human PBLs with RevM10 vector also conferred resistance to HIV-1 infection.

Dominant negative HIV-1 Tat mutants have also been studied [46, 47]. Unlike the HSV VP16 or Rev, however, transdominant Tat was generated with mutations in the RNA binding/nuclear localization domain, and not the activation domain [46, 47]. A possible mechanism of action is sequestration of cellular factors involved in Tat transactivation.

Structural proteins: Gag, Env and Vpx. Trono *et al.* exploited the fact that HIV-1 gag gene products exist in

highly multimerized forms in the mature virion and showed that several gag deletion mutants could interfere with the formation of infectious viral progeny [48]. Such a mutated gag sequence was subsequently expressed in a retrovirus vector for gene delivery [49].

The HIV envelope protein is another target. One mutant, termed 41.2, substitutes Glu for Val at the second amino acid of the transmembrane protein gp41 [50]. Stable cell lines expressing the HIV envelope protein are difficult to establish because of the cytopathicity induced by interaction of gp120 with cell surface CD4. Using a cell line that expressed 41.2 upon HIV infection, Buchschacher *et al.* [50] showed that these cells not only resisted cytopathic effect (CPE) caused by wild-type HIV-1 but also had decreased infectious virus production. 41.2 was presumed to exert its dominant negative effect by interfering with the hydrophobic interaction of the envelope glycoprotein multimer and the receptor complex. Steffy and Wong-Staal [51] created a transdominant mutant of the HIV-2 envelope protein by deleting part of the CD4 binding region of gp120. The truncated protein no longer bound CD4 and interfered with production of infectious virus from a wild-type genome. Such a transdominant Env protein may be advantageous if the gp120–CD4 interaction itself has immunosuppressive consequences.

Matsuda *et al.* have explored the intriguing strategy of 'virus specific inhibitory molecules' (VSIM) [52]. They generated an HIV-1/HIV-2 chimeric virus by inserting the HIV-2 vpx gene into the *vpr* coding region of HIV-1 HXB2. Vpx-chimeric virus produced by transfection into COS-7 cells was unable to replicate in SupT1 cells [52]. Cotransfection of the vpx chimeric virus DNA with HIV-1 HXB2RU, which has an intact vpr gene, also showed some inhibitory activity. This trandominant property of Vpx may derive from two features of the protein: it is virion-associated and it has significant homology with Vpr. However, both the exact mechanism of inhibition and the generalizability of this result await elucidation. For example, LeGuern and Levy have reported a potentially contradictory result: cloned T-cell lines chronically producing HIV-2-UC1 were superinfectable with HIV-1 [53].

Limitations of transdominant proteins. The above transdominant negative mutant proteins have potential limitations. First, since they function by suppressing viral expression or interfering with virion maturation, their effects are confined to postintegration events. Another problem of theoretical concern is that these foreign viral proteins are anticipated to undergo intracellular processing into antigenic peptides that are presented to class I MHC, and the resulting CTL response may destroy the transgene-expressing cells. Thus, an effect that provides the underlying allure of gene-transfer-based vaccines may be a liability for antiviral gene therapy. In this regard, inhibitory genes that are derived from cellular proteins or are expressing RNA (decoys, antisense, ribozymes) rather than foreign protein molecules may be preferable.

Cellular targets

Soluble CD4. Shortly after the first description of HIV as the cause of AIDS, the CD4 molecule was established to be the HIV receptor on inducer/helper T cells and monocyte/macrophages [54]. Subsequent intensive studies of the molecule drew impetus from the hope that understanding the structure and function of CD4 would disclose avenues for therapeutic intervention. Three years later, several groups reported that exogenous recombinant sCD4 can indeed block HIV-1 infection in cultured T cells [55–58]. As the normal function of CD4 in the immune system involves class II MHC recognition, an early concern was that expression of sCD4 might interfere with class II specific T-cell interactions. This question was recently addressed by a transgenic mouse model [59] in which constitutive expression of 100 μg monovalent or 20 μg decavalent murine sCD4 per ml of serum did not affect the response of murine $CD4^+$ cells to allogeneic or anti-CD3 antibody stimuli. Furthermore, the ability of T helper cells to mediate antibody responses *in vivo* was unperturbed.

In early studies, Morgan and colleagues showed inhibition of HIV-1 infection of human T cell lines following transfer of the sCD4 gene [58] via a retroviral vector. More recently, a $sCD4^+$ immunoglobulin fusion protein was placed under the control of the HIV-1 long terminal repeat (LTR) to achieve expression inducible by Tat, the viral transactivator. However, virally induced expression has some disadvantages: sCD4 can no longer be secreted from non-HIV target cells (e.g. fibroblasts) and the potential of blocking the infection cycle before proviral integration by secretion from the same cell is lost.

Moreover, sCD4 appears to have intrinsic shortcomings unrelated to problems of gene delivery. Infusion of recombinant sCD4 protein directly into HIV-infected patients failed to show efficacy in phase I clinical trials, despite the achievement of high plasma levels of recombinant protein [61]. Marked resistance of clinical isolates to the neutralizing activity of sCD4 was eventually documented: 200–2700 times more sCD4 was required to neutralize clinical isolates than to neutralize *in vitro* passaged strains of HIV-1 [62–64]. These studies illustrate how a conceptually promising strategy can founder in the transition from the bench to the bedside.

Sequestration of the viral envelope. Buonocore and Rose [65, 66] devised an intriguing modification of human CD4 with therapeutic potential by incorporating into the molecule a tetrapeptide endoplasmic reticulum (ER) retention signal, KDEL [67]. Cotransfection of this gene with gp160 resulted in tethering of gp120 in the ER, blocking of surface expression/secretion of gp120, and prevention of syncytia formation in $CD4^+$ HeLa cells. Transport of wild-type CD4 was not affected. More recently [66], a retrovirus vector LXSN [68] was used to deliver this modified CD4 molecule (sCD4-KDEL) into H9 cells. Expression of sCD4-KDEL was driven by an internal HIV-1 LTR and hence was Tat-inducible [69]. Transduced, cloned H9 cell lines subsequently resisted productive infection by HIV-1 MN. Viral entry and

proviral integration did occur since some of the MN-challenged cells stained positively for HIV-1 antigens, but cell surface expression was not seen. It remains to be seen whether this 'intracellular trap' approach can work in primary T cells and if it will have the same shortcoming as soluble CD4: markedly less efficacy against primary isolates. A similar strategy that diverts gp160 to lysosomes has recently been published [70].

Intracellular antibodies. An alternative approach to sequester the HIV-1 envelope was described by Marasco *et al.* [71]. These investigators developed a single chain antibody [72] capable of binding the nascent HIV-1 envelope protein in the ER. Since single immunoglobulin chains are not exported without their partner chains, this molecule is retained in the ER, with or without a KDEL signal; there, it blocks transition of the envelope-antibody complex to the cell surface [71]. Cells cotransfected with HIV-1 and the single chain antibody produced similar levels of Gag protein but fewer infectious HIV-1 particles. Recently, Pomerantz *et al.* (personal communication) developed a single chain antibody against Rev which trapped the Rev protein in the cytoplasmic compartment and inhibited HIV expression.

Interferon-inducible genes. The 210 nucleotide RRE located in the *env* coding region of HIV-1 structural gene transcripts has an elaborate, multi-looped secondary structure. Binding of Rev to this structure allows expression of HIV-1 structural proteins by enhancing the stability and transport of unspliced and singly spliced viral mRNAs [39, 73]. By screening a human cDNA expression library for proteins that bind to the RRE, Pavlakis *et al.* discovered that the previously known interferon-inducible gene 9-27 encodes a protein that binds specifically to hairpin loops 3, 4 and 5 of the RRE [74]. Cotransfection of DNA encoding this 125 amino acid protein (now designated RBP9-27) with an HIV-1 proviral clone resulted in specific, dose-dependent inhibition of unspliced (Rev-dependent) HIV-1 RNA expression and viral structural protein production. Specificity was further established by demonstrating that expression from a base-substituted, Rev-independent Gag gene [75] was not affected by RBP9-27. How native expression of this cellular Rev antagonist figures into the host response to HIV-1 infection or in the overall *in vivo* antiviral effects of interferon remains unclear. Nevertheless, the transfer of genes for inhibitory cellular factors could turn out to be a plausible therapeutic strategy.

RNA decoys

The decoy strategy seeks to sequester viral nucleic acid-binding regulatory proteins through overexpression of their cognate RNAs. The two common targets are Tat and Rev. Gilboa and colleagues inserted the HIV-1 TAR [76–78] and RRE [79] sequences into a retroviral vector with an internal pol III ($tRNA^{met}$) promoter in concert with a double copy expression cassette to achieve constitutively high 'housekeeping gene' levels of expression. CEM SS cells transduced with these TAR and RRE constructs specifically exhibited reduced HIV expression and replication. Site-directed mutations showed that the integrity of the TAR stem-loop structure was important for the decoy function [77]. One potential concern for the decoy strategy is that cellular factors that bind TAR or RRE will also be sequestered. In an attempt to increase the effectiveness of the TAR decoy strategy, as well as minimize its potential toxicity, Lisziewicz and colleagues in Gallo's laboratory expressed a polyTAR sequence driven from the HIV-1 LTR [80]. Recent experiments carried out by this group showed long-term protection of a T-cell line from HIV replication [81].

As discussed above for transdominant mutants, RNA decoys are only effective after the integration step, a drawback that hematopoietic stem cell therapy would not obviate.

Antisense RNA and ribozymes

Ribozymes and antisense RNA utilize the specificity of Watson–Crick base pairing to interfere with gene expression in a sequence-specific fashion.

Antisense RNA. The antisense RNA strategy mimics a naturally occurring mechanism of gene regulation in prokaryotes [82]. Zamecnik and Stephenson [83] first took this approach using synthetic oligonucleotides directed at a retrovirus (Rous sarcoma virus). Extensive literature exists on the subsequent application of antisense oligonucleotides to viral diseases [84–88] (reviewed in ref. 89); we will confine our discussion here to studies that have employed stable expression of antisense RNA to inhibit the replication of HIV-1. Initial studies showed limited efficacy with pol II-promoted transcripts complementary to tat, rev, vpu [90, 91], gag [92], and the primer binding site [93].

Chatterjee *et al.* [94] demonstrated the utility of using a recombinant adeno-associated virus (AAV) vector to transduce an HIV-1 antisense construct into human T cells (the properties of AAV are enumerated in a succeeding section). The antisense RNA in this study was complementary to a 63 bp sequence in the HIV-1 LTR including the TAR sequence and part of the polyadenylation signal; thus the sequence is present at both 5' and 3' ends of all HIV-1 transcripts. In CD4 cells, specific inhibition of LTR-CAT expression was observed and 70–90% reduction was seen in single round virus production after transfection of HIV-1 HXB2. Transfection of a clone of simian immunodeficiency virus (SIV) (which has some LTR homology with HIV-1) resulted in only slightly less inhibition. Diminished accumulation of HIV-1 RNA was demonstrated in antisense-expressing clones. Transduction of a human T-cell line with the antisense vector resulted in a three log reduction in virus titer after challenge. A potential caveat of this study is that a non-specific, general anti-retroviral effect was not ruled out.

Ribozymes. Ribozymes are antisense molecules with an edge. Because of their catalytic properties, ribozymes can theoretically be effective at much lower

concentrations. The greater constraint inherent in the rules for substrate selection should also minimize potential toxicity due to non-specific gene inhibition.

Sarver and Rossi described the first use of a hammerhead ribozyme as an anti-HIV agent in 1990 [95]. In this study, HeLa-CD4$^+$ cells stably expressing a ribozyme targeting the gag sequence expressed reduced p24 levels compared with the non-ribozyme expressing cells upon infection with HIV. Since then, several groups have reported design and functional study of hammerhead ribozymes targeting other HIV sequences including vif [96], integrase [97] and the leader sequence (+133, NL43, [98]), all reporting various delays or reduction in virus expression. We reported that a hairpin ribozyme derived from the negative strand satellite RNA of the tobacco ringspot viroid [-sTRSV] and designed to cleave HIV-1 RNA in the 5' leader sequence (positions +111/112 from the cap site in IIIXB2) suppressed virus expression in HeLa cells cotransfected with proviral DNA from diverse HIV-1 strains, suggesting that the ribozyme was effective in blocking virus expression from pre-existing proviral DNA [99, 100]. Furthermore, a disabled ribozyme unaffected in its substrate binding ability had minimal antiviral effect, indicating that the primary effect of the ribozyme is indeed due to its cleavage capability. Subsequently, human T-cell lines [101] and primary T cells obtained from normal human peripheral blood (Leavitt *et al.*, unpublished) transduced with retroviral vectors containing this hairpin ribozyme exhibited complete long-term resistance to the challenge of HIV-1 clinical isolates.

Potentially, ribozymes can target the replication cycle during both the early (prior to provirus integration) and late (subsequent to provirus integration) phases by cleaving incoming virion RNA, as well as transcribed genomic and subgenomic mRNAs [102]. In the study of Yamada *et al.* [101] we showed that expression of the ribozyme significantly decreased (50–100-fold) the efficiency of incoming virus to synthesize viral DNA. These results indicate that the transfer and expression of the ribozyme gene interfered with both early and late events of the HIV replication cycle, resulting in potent synergistic inhibition of virus production. This makes the ribozyme a unique molecular genetic intervention for the treatment of HIV infection. Figure 1 compares the different steps of the HIV replication cycle that the various therapeutic genes target. Joshi *et al.* [103] employed an HIV-1 leader sequence ribozyme driven by the TK-TAR fusion promoter as part of the 3' non-translated region of the neo gene. Retrovirus-mediated gene transfer of this construct into human T cells also conferred long-term (22 days) protection. Although the use of inducible expression would diminish the

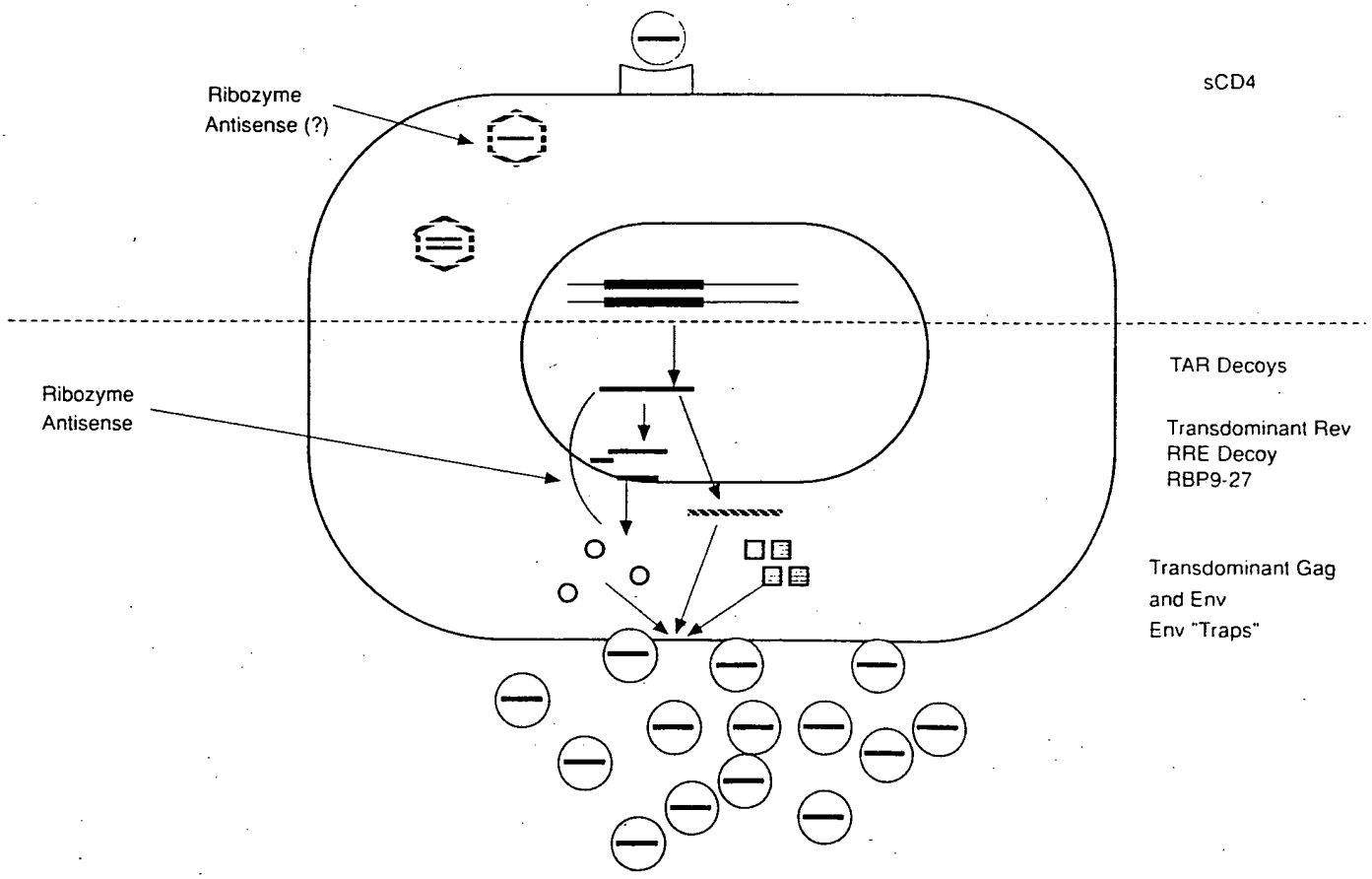

Figure 1 Targets for anti-HIV therapy. A scheme of the replication cycle of HIV, indicating whether the various genes target steps before or after provirus integration.

possibility of toxicity for the ribozyme expressing cells, the above-discussed ability to cleave afferent viral genomes is also lost.

RAC-approved clinical protocols

Two clinical protocols involving intracellular immunization with virus inhibitory genes – the RevM10 transdominant protein and the HIV-1 leader sequence hairpin ribozyme – have been approved by the NIH Recombinant DNA Advisory Committee (RAC). A third protocol involving the transfer of syngeneic gene-marked cells from uninfected individuals to their HIV-1 infected monozygotic twins [102] was also approved. For the latter trial, the patients will be selected from a cohort of identical twins discordant for HIV-1 infection identified by Blaese *et al.* [104]; this unique population may prove valuable for additional studies beyond gene-marking.

Nabel *et al.* [105] received RAC approval in June 1993 to transduce $CD4^+$ T cells with RevM10 in HIV-1 infected individuals (see information on transdominant proteins above); zidovudine therapy will also be administered. Our laboratory received approval in September 1993 to initiate a similar trial using LNL6 to transfer an HIV-1 leader sequence hairpin ribozyme gene into autologous $CD4^+$ T cells[106]. The purpose of these trials is to track the gene-altered cells in the *in vivo* milieu of HIV-1 infected individuals. As such, the most important endpoint for both studies will be determination of safety and the relative survival of the two infused cell populations: PBLs transduced with vectors bearing the therapeutic gene (RevM10 or the hairpin ribozyme) and PBLs transduced with a control vector. $CD4^+$ T cells will be harvested from HIV-1 positive patients, stimulated with anti-CD3 monoclonal antibodies, transduced by retroviral vectors and then expanded in interleukin-2 prior to reinfusion. Since these stimulatory conditions are ideal for promoting the outgrowth of HIV-1, a combination of nevirapine and CD4-*Pseudomonas* exotoxin [107, 108] will be included during the *ex vivo* manipulations. Nevirapine is an HIV-1 specific, non-nucleoside reverse transcriptase (RT) inhibitor [107] that will not block infection by the MMLV-based vectors to be used in this study.

Greenberg *et al.* have already initiated the first gene transfer trial in HIV-infected individuals: adoptive transfer of gene-marked $CD8^+$ T-cell clones into patients undergoing bone marrow transplantation for HIV-1 related non-Hodgkins lymphomas [109]. In September 1993, this team received additional approval for the use of the same approach in HIV-1 infected patients without lymphoma (September 1993 RAC meeting). As discussed earlier, these studies incorporate an additional safety feature into the retroviral vector: a suicide gene which is a fusion product of the hygromycin phosphotransferase gene and the HSVtk gene (see Immunotherapy above). The principal endpoints will be determination of safety, assessment of survival (by PCR as in the other trials) of the marked cells, preliminary observations on HIV-1 disease activity and, if necessary, assessment of the ability of ganciclovir administration to ablate the transferred cells. Finally, immune modulation by direct injection of a retroviral vector expressing gp160 has been approved for phase I human trial. In one study [22], autologous skin fibroblasts from patients with HIV-1 infection will be cultured *ex vivo*, transduced by a retroviral vector bearing the HIV-1 IIIB envelope gene, lethally irradiated and then infused in an attempt to bolster immunity. Endpoints will include monitoring of T-cell counts. A second clinical study from the same group involving direct injection of the vector intramuscularly has recently been approved by the RAC [23].

Future directions

Coping with resistant mutants

Although there is no evidence so far of the generation of escape mutants in intracellularly immunized cells, in principle, escape mutants could arise to circumvent the action of any therapeutic transgene, as in the case of chemotherapeutic compounds. The problem is not, however, insurmountable. First, the mutational capacity of the virus is not unlimited. Some regions are highly conserved among diverse strains and extensive mutations in them may lead to a loss in infectivity. The leader sequence targeted by the hairpin ribozyme [99, 100] may be a good example. Therefore, the proper choice of a target site may be important. Second, the long-term benefits of gene therapy make it more feasible to intervene early after infection, at a time when there is less virus replication and therefore less chance of generating escape mutants. Finally, a combination of targets and/or approaches (e.g. multi-target ribozymes, TAR/antisense, ribozyme/RevM10 etc.) will further minimize resistance.

Alternative gene transfer techniques

Murine retrovirus vectors have been the vehicle of choice for gene delivery for a variety of systems because of their efficiency, safety and stable integration for persistent expression [110]. However, there are limitations for their use in HIV infection: murine retroviruses only infect dividing cells, whereas one of the major target cells for HIV is the non-dividing monocyte/macrophage. Murine retroviruses also do not yield to simple concentration procedures, often making it difficult to transduce primary PBLs at a sufficiently high multiplicity of infection. Finally, the remote possibility of contamination with replication-competent helper virus remains a finite risk for use of these vectors. Therefore, much effort is expended in the development of alternate means of gene delivery. The following selectively reviews some of these approaches.

HIV-based retroviral vectors. HIV-1 or HIV-2-derived replication-defective vectors and packaging cell lines have obvious advantages for HIV gene therapy: (a) specific targeting of the relevant $CD4^+$ cell populations; (b) the ability to transduce non-dividing cells [111], a property not generally shared by conventional retroviral vectors; and (c) the potential to undergo *in vivo* rescue by HIV-1 and be delivered to additional $CD4^+$ cells.

However, the CD4+ cell target specificity of HIV could be a drawback for stem cell transduction (although the capacity of the HIVs to infect these cells is still unclear). A possible alternative would be to pseudotype such particles with heterologous envelopes conferring a broader host range; pseudotyping with more stable envelopes might also allow the concentration of vector supernatants to increase the titer [112].

Sodroski's group first reported identification of sequences required for HIV-1 packaging [113] and demonstrated transfer of the neo^r gene into a human T-cell line by a replication-defective HIV-1 based vector [114, 115]. The marker gene was driven by an internal promoter and contained a polyadenylation signal in addition to that in the 3'LTR. This neo^r cassette was flanked upstream by the HIV-1 5'LTR plus a portion of gag and downstream by the 3'LTR. Reversal of the neo^r cassette led to higher titer, presumably because more full length genomes were made without the competing upstream poly(A) signal. Structural genes were supplied *in trans* via cotransfection with an HIV-1 provirus that was intact except for a deletion of 19 bp between the 5' splice donor site and the gag initiation codon. Low levels of helper virus were observed in this system; subsequent separation of the structural genes into distinct expression plasmids reduced this to undetectable levels.

Other groups have designed HIV-1 based retroviral vectors that are Tat-inducible [116] or that use heterologous promoter constructs [117]. Recent studies by Lever *et al.*[118] suggest that, as with avian retroviruses, a 3' viral element could be important for HIV-1 packaging.

An HIV-2 based vector/packaging line system may be preferable in that it would be impervious to HIV-1-specific interventions such as HIV-1 targeted antisense RNA and ribozymes, but the vector would still be rescuable by HIV-1. In this regard, HIV-1 has been shown to cross-package SIVmac genomic RNAs (pseudotyping at the RNA level) as efficiently as those of HIV-1 [119]. Although a deletion in HIV-1 between the major 5' splice donor and the gag ATG appears to attenuate packaging, the exact nature of the packaging signal for both HIV-1 and HIV-2 remains uncertain [120]. Some 5' portion of the gag gene is necessary for optimal (i.e. psi$^+$) packaging for most retroviruses [110]. However, HIV gag contains instability (INS) elements [121] which are normally overridden by the RRE/Rev interaction. Incorporation of Rev/RRE genes will also increase the chance of recombination between vector and helper viral sequences. A possible solution is to incorporate gag sequences with the INS elements inactivated [121].

Adeno-associated virus (AAV). Chatterjee *et al.* [94] have published encouraging data for describing the use of AAV as a gene transfer vehicle for HIV-1 target cells (see section on Antisense for experimental details). Properties of AAV that render it potentially useful, especially as a gene therapy vector for hematopoietic stem cells, have been comprehensively reviewed elsewhere [122]. The advantages of this replication-defective DNA virus-derived vector that are most relevant for HIV applications can be briefly summarized as follows: (1) current recombinant AAV vectors have no potential for homologous recombination; (2) they have high transduction efficiency for hematopoietic cells including progenitor cells [123]; (3) vector can be easily concentrated from culture supernatant; (4) the parental virus is non-pathogenic; (5) lack of endogenous promoter activity reduces risk of promoter interference; (6) non-dividing cells (e.g. monocytes, stationary T cells and non-activated stem cells) can be transduced. Disadvantages include a limit of 4.7 kb for the insertion of therapeutic sequences, the risk of adenovirus contamination and the possibility of excision of the integrated gene.

Non-vectored delivery. The safest means of gene delivery is to completely bypass the use of virus vectors. A number of methods, including direct DNA injection with or without lipofectin agents, ballistic gold particles [124] and an adenovirus-polylysine-transferrin complex conjugate [125] have been shown to be able to introduce genes into cells, with varying degrees of efficiency. However, the suboptimal gene transfer efficiency and/or lack of integration of the transgene presently limit the utility of these methods for antiviral therapy in the clinical setting.

The ultimate goal for the global treatment of HIV infection is the development of an injectable vector, as *ex vivo* manipulation of cells would be a procedure only accessible and affordable to a privileged minority. Unfortunately, this may be in the distant future.

Hematopoietic stem cell gene therapy

Although the loss of CD4+ cells is the major proximate cause of the immune deficiency of AIDS, other cells such as macrophages, dendritic cells and brain glial cells are also infected [126]. Moreover, natural senescence of the transduced cells would periodically require repeated, large-scale transfers of gene-altered T cells, a process that is too logistically complex, expensive and dependent on specialized expertise to be practical. Although the initial clinical trials to examine survival of transduced peripheral blood T cells will yield invaluable information for the feasibility of particular gene therapies for HIV or even confer limited therapeutic benefits on the patients, gene transfer into pluripotent hematopoietic stem (progenitor) cells would be a preferred strategy for achieving sustained immune reconstitution [127]. Stem cells possess two important characteristics [128] for this purpose: the ability to give rise to all hematopoietic lineage and the capacity for self-renewal. Stable transduction of stem cells might thus allow the permanent repopulation of all hematopoietic cell lineage of the immune system with intracellularly immunized cells.

The technical difficulties of this approach remain substantial, but recent developments in stem cell biology provide grounds for optimism [129]. Efficient hematopoietic reconstitution of mice with genetically altered stem cells has been reproducibly successful [130–136]. The biology of the human stem cell is less

well delineated than the murine system [137, 138], but engraftment of lethally irradiated baboons with autologous $CD34^+$ cells has been achieved [139] and human cord blood has been used to successfully engraft an HLA-identical sibling [140]. Numerous patients have now undergone hematopoietic rescue with autologous peripheral blood stem cells (with or without simultaneous bone marrow rescue) following marrow ablation [141–143]. McCune *et al.* have developed a SCID-hu Thy/Liv mouse model [144], which may be appropriate for the study of human stem cells. The mice can be reconstituted with anti-HIV therapeutic gene-transduced human stem cells and subsequently challenged *in vivo* with HIV [145].

Truly pluripotent hematopoietic cells ($CD34^+/CD38^-$) normally represent probably less than $1/10^4$–10^5 bone marrow cells and $1/10^6$ peripheral blood lymphocytes. Advances in procuring sufficiently pure populations of human $CD34^+$ cells include the development of immunomagnetic enrichment [146], immunoabsorbent columns [147], delineation of complex cytokine and tissue culture requirements [148] and the realization that increased amounts of such cells can be retrieved from human umbilical cord blood [149] and from the blood of patients in the recovery phase subsequent to high-dose cyto-ablative chemoradiotherapy and/or hematopoietic growth factor administration [150–152].

A human stem cell gene therapy trial for ADA deficiency received RAC approval in February, 1992 [153], and the first patient was treated with gene-corrected autologous stem cells derived from peripheral blood in May 1993 under the direction of Blaese and colleagues at the NIH.

Whether human hematopoietic stem cells may already be infected by HIV *in vivo* is still a controversial issue [154–157] and should be re-examined with current highly sensitive technology. A positive outcome will complicate, though not necessarily prohibit stem cell gene therapy for the infected. The blood–brain barrier, a barrier the virus does not respect, represents an additional hurdle of uncertain importance for HIV gene therapy. The pathogenesis of HIV-associated neurological syndromes remains particularly unexplored [158]. Brain glial cells and monocytes/ macrophages do presumably arise from blood-derived hematopoietic progenitors and appear to be the principal target cells [159]. Nevertheless, it remains uncertain whether the often disabling outcomes (e.g. AIDS dementia) of intracerebral infection, an early and consistent event in the natural history of HIV disease, would be reversible or preventable, even if stem cell therapy effective outside the central nervous system is developed. Although improvements in cognitive function reported with Zidovudine therapy are encouraging [158], the existence of previously infected cells in the brain could suffice to carry out a program of irreversible damage.

Conclusion

We may anticipate that the number of investigations of gene therapies for HIV will continue to escalate. In the absence of truly informative animal models, it remains crucial, then, to establish standards of experimental proof that should ideally be met before a therapeutic gene is pursued. Efficacy, potency, specificity and lack of cellular toxicity are all important criteria. The full range of functional properties demanded of immune system cells *in vivo* cannot be comprehensively assayed *in vitro*. There may be secondary, even useful, viral interference effects in addition to specifically designed mechanisms; however, investigators should design experiments to show that gene transfer and selection have not merely created a 'subtly sick' and therefore less permissive cell. Cooperative, unbiased comparisons of interventions originating in different laboratories will also be important for deciding which interventions merit the risk and expense of clinical trials.

The potential of human gene therapy is only now beginning to be realized for the first human applications; ADA gene therapy can now be counted a definite, if still qualified, success; the results of the stem cell trial now in progress for this disorder are eagerly awaited. The results of other early applications remain less definitive, although they have yielded invaluable information. Application of human gene therapy to the HIV problem, with its attendant pressures to balance the very real urgency of an unchecked pandemic with the critical need for controlled, stepwise scientific inquiry, will bring unprecedented challenges. We are indeed entering an exciting era of investigation; overselling the promise of this emerging technology or circumventing standards of rigorous investigation will benefit neither patients nor scientists.

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